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a Saxitoxin-Binding Protein from the Bullfrog

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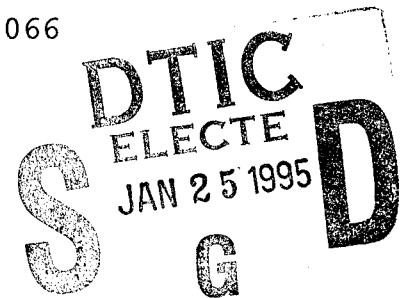
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13. ABSTRACT (Maximum 200 words) Saxitoxin (STX), a potent neurotoxin, is the causative agent of paralytic shellfish poisoning in humans. The North American bullfrog, <i>Rana catesbeiana</i> , contains a plasma protein called saxiphilin that binds STX with high affinity and specificity. Inasmuch as saxiphilin may be useful as a detection reagent or an antidote for STX, we have investigated the biochemical properties of saxiphilin and cloned cDNA encoding this protein. Native saxiphilin is a polypeptide of 826 amino acid residues (Mr = 90,818) that contains one binding site for [ <sup>3</sup> H]STX per molecule with an equilibrium dissociation constant of K <sub>D</sub> ≈ 0.2 nM. The amino acid sequence of saxiphilin, deduced from cDNA isolated from bullfrog liver, exhibits substantial homology to members of the transferrin family of Fe <sup>3+</sup> -binding proteins. However, biochemical and immunochemical analyses confirm that saxiphilin is a unique protein that is not derived from bullfrog serum transferrin. Also, saxiphilin does not bind Fe <sup>3+</sup> which implies that saxiphilin is probably not involved in iron metabolism. The mechanism of [ <sup>3</sup> H]STX binding to saxiphilin, including the pH-dependence and temperature-dependence, has been characterized in detail. Recombinant saxiphilin has been expressed in insect cells using a baculovirus vector and the STX-binding site has been localized to the C-lobe domain of the protein.				
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*Edward M. Magallon* 9/21/94  
PI - Signature Date

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## MIDTERM REPORT [DAMD17-93-C-3069]

**Principal Investigator and Author:** Edward G. Moczydlowski, Ph. D.

**Title of Project:** Cloning and Functional Analysis of Saxiphilin, a Saxitoxin-Binding Protein from the Bullfrog

**INTRODUCTION:** Saxitoxin (STX), a small heterocyclic molecule containing two guanidinium groups (Fig. 1), is a potent blocker of voltage-dependent  $\text{Na}^+$  channels that function in the electrical excitability of nerve and muscle. This neurotoxin is known to be produced by certain species of marine dinoflagellates and freshwater cyanobacteria (1). STX and its homologs are the agents responsible for paralytic shellfish poisoning (PSP) in humans which results from consumption of contaminated shellfish.

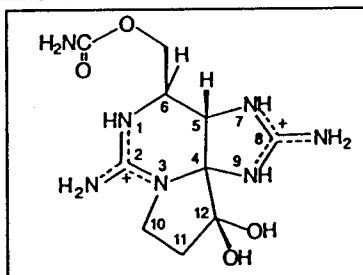


Fig. 1. Structure of saxitoxin.

In the course of radioligand binding studies using [ $^3\text{H}$ ]STX to monitor the distribution of  $\text{Na}^+$  channel proteins in bullfrog (*Rana catesbeiana*) tissues, Moczydlowski and coworkers (2) observed the presence of an unusual binding site for STX in soluble extracts of frog skeletal muscle. Since functional  $\text{Na}^+$  channels are integral membrane proteins, the molecular identity of the component responsible for the soluble [ $^3\text{H}$ ]STX binding activity in frogs became a question of considerable interest. To pursue this question, a biochemical characterization of the soluble [ $^3\text{H}$ ]STX-binding component was carried out (3). This work showed that plasma and other bullfrog tissues contain a soluble protein with an apparent molecular weight of 74,000 (estimated by gel permeation chromatography) that binds STX with an equilibrium dissociation constant of  $\sim 0.2$  nM. Competition binding experiments showed that this binding site was chemically specific for STX and a diverse group of natural and synthetic derivatives of STX. The protein responsible for this STX-binding activity was named saxiphilin to distinguish it from  $\text{Na}^+$  channel proteins. In 1991, Li and Moczydlowski (4) reported the purification of saxiphilin from bullfrog plasma. Native saxiphilin consists of a single polypeptide with an apparent molecular weight of  $\sim 89,000$  as determined by SDS-PAGE. Sequencing of the amino-terminus and five tryptic fragments of the purified protein revealed that saxiphilin exhibits sequence similarity to members of the transferrin family of  $\text{Fe}^{3+}$ -binding proteins.

Transferrins are soluble monomeric proteins with a molecular weight of  $\sim 77,000$  that bind  $\text{Fe}^{3+}$  with high affinity ( $K_D \sim 10^{-20}$  M) in a bicarbonate-dependent fashion (5). Three distinct

transferrin proteins have been identified in mammals: serum transferrin, lactoferrin, and melanotransferrin. Serum transferrin is secreted by the liver and several other tissues. Its function is to bind and transport  $\text{Fe}^{3+}$  to cells via receptor-mediated endocytosis that involves the cell-surface transferrin receptor. Serum transferrin is an essential cellular growth factor. Lactoferrin is present in milk and other secretions and serves as an antimicrobial defense mechanism by keeping the concentration of free  $\text{Fe}^{3+}$  very low, thus limiting the growth of microorganisms (6). Melanotransferrin was originally described as a tumor antigen present on the surface of melanoma cells (7); its exact function is unknown. The finding that bullfrog saxiphilin is structurally related to transferrin raised several questions: Is saxiphilin a derivative of transferrin or identical to transferrin itself? Does saxiphilin bind metal ions such as  $\text{Fe}^{3+}$  and conversely, do transferrins bind STX? What is the primary sequence of saxiphilin and what is its physiological function?

Aside from these important basic questions of cellular biochemistry, the discovery of saxiphilin also raises the possibility that this protein might have useful applications in toxicology and pharmacology. For example, a soluble protein that specifically binds saxitoxin with high affinity might be useful in the assay and detection of STX and STX derivatives in contaminated shellfish and other biological samples. Also, such a protein might be able to function as a toxin scavenger in animals exposed to saxitoxin. Studies of the effectiveness of saxiphilin in antagonizing saxitoxin poisoning in animal models could yield valuable information on the mechanism of action of STX at the whole-animal level. Such investigations might ultimately lead to the development of a human antidote for STX. Furthermore, the availability of an antagonist binding-protein for STX might lead to new applications of STX in neuroscience or clinical applications of STX as a local anesthetic. In pursuing these ideas, it is clear that much needs to be learned about the structure and function of saxiphilin before such applications become practical. Also, methods must be developed for large-scale production of the pure protein to permit the systematic evaluation of such applications.

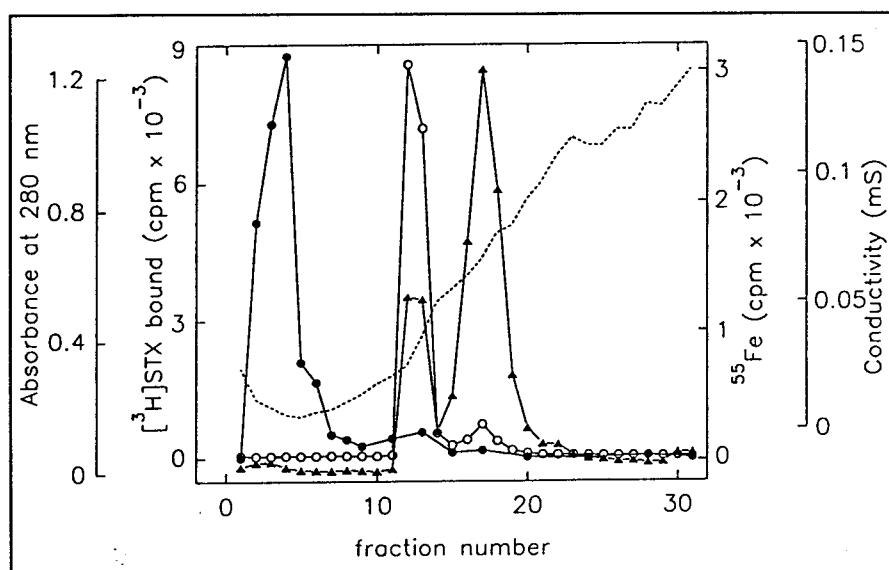
With these issues in mind, the U.S. Army Medical Research and Materiel Command has funded a contract grant on the "Cloning and Functional Analysis of Saxiphilin, a Saxitoxin-Binding Protein from the Bullfrog." The primary goal of this project as outlined in the statement of work (USAMRMC Log. No. 90296008) is as follows: "The contractor shall furnish all equipment, personnel, facilities and supplies required to clone and express bullfrog saxiphilin in a recombinant system, and to develop large-scale preparation procedures for research quantities of the recombinant saxiphilin for possible use as a research tool or a countermeasure against saxitoxin."

This report represents a comprehensive summary of all work conducted on this project to midterm. Our efforts have yielded new basic information on the structure and biochemical properties of saxiphilin. In particular, a cDNA encoding saxiphilin has been cloned from bullfrog liver, and the exact structural relationship of saxiphilin to transferrin proteins has been established. Recombinant saxiphilin has been successfully expressed in cultured insect cells using a baculovirus vector. Efforts are currently under way to monitor and increase the yield of recombinant protein. These achievements indicate that the objectives of the grant are feasible and should be accomplished in due course. Most of the results reported here in abbreviated form are described

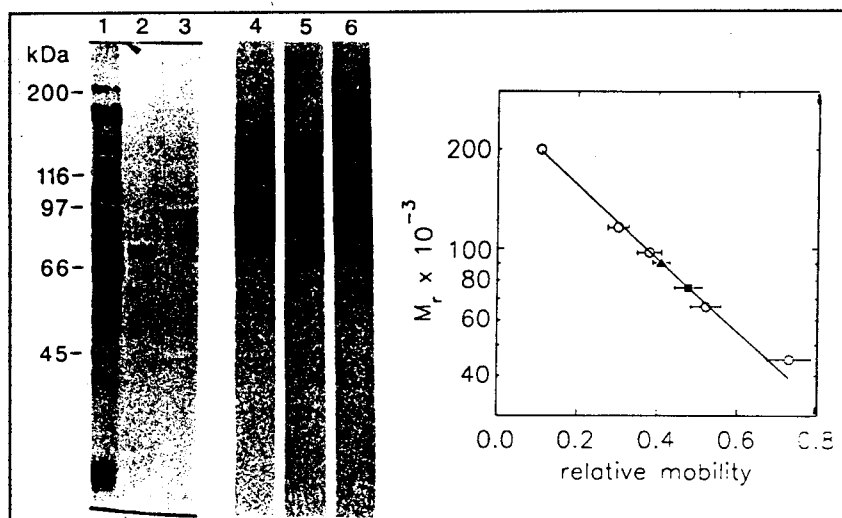
in detail in three recent publications (8-10) that have resulted directly from this grant. These publications include a complete description of methodology and data analysis. Interested readers are referred to these sources for additional details.

## BODY OF REPORT:

**1. Direct Confirmation that Saxiphilin is Biochemically and Functionally Distinct from Bullfrog Serum Transferrin.** Since partial amino acid sequences of native saxiphilin displayed homology to transferrin proteins (4), it was important to establish the exact relationship of saxiphilin to bullfrog serum transferrin. This objective was accomplished by purifying both proteins from plasma and comparing their properties. To monitor serum transferrin during its purification, 10 ml of bullfrog plasma was labeled with  $^{55}\text{Fe}^{3+}$  by addition of 10 mM  $\text{NaHCO}_3$  and 15  $\mu\text{M}$   $\text{Fe(NTA)}_2$  premixed with 45  $\mu\text{Ci}$  of  $^{55}\text{FeCl}_3$ . These labeling conditions are known to promote the binding of  $\text{Fe}^{3+}$  to apotransferrin. The  $^{55}\text{Fe}$ -labeled plasma sample was then subjected to gel filtration chromatography on a 2.5 x 50 cm column of Sephadex G-200 equilibrated with 100 mM Tris-HCl, 1 M NaCl, pH 7.8. and eluted with the same buffer. A peak of soluble protein-bound  $^{55}\text{Fe}$  eluting after the void volume was pooled, dialyzed and further subjected to anion exchange chromatography on a column of DEAE-Sephadex. Figure 2 shows the elution profile of this column as monitored for total protein by absorbance at 280 nm,  $^{55}\text{Fe}$  and assay of  $[^3\text{H}]\text{STX}$  binding. The results of Fig. 2 show that  $[^3\text{H}]\text{STX}$ -binding activity and protein-bound  $^{55}\text{Fe}$  in frog plasma are separable activities. Saxiphilin, being a basic protein ( $\text{pI} \sim 10.5$ ) does not adsorb to the cationic DEAE resin and elutes first from this column as noted by the large peak of  $[^3\text{H}]\text{STX}$  binding. In contrast, serum transferrin is a more acidic protein and elutes from the DEAE column at higher ionic strength as noted by a large peak of  $^{55}\text{Fe}$  centered near fraction 13 in the profile of Fig. 2.



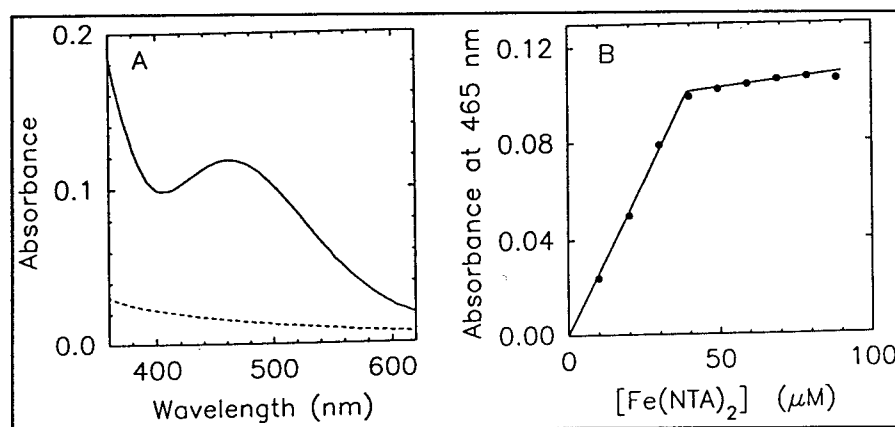
**Fig. 2.** Separation of  $[^3\text{H}]\text{STX}$  and  $^{55}\text{Fe}$ -binding activities in bullfrog plasma. (Δ) protein absorbance at 280 nm; (●) specific binding of  $[^3\text{H}]\text{STX}$ ; (○)  $^{55}\text{Fe}$ ; (---) conductivity. Experimental details are given in Ref. (8).



**Fig. 3.** SDS-Page and immunoblots of whole plasma, transferrin and saxiphilin. A, Lanes 1, 2, and 3, SDS-PAGE of 10  $\mu$ g of bullfrog plasma, 1  $\mu$ g of bullfrog transferrin and 1  $\mu$ g of bullfrog saxiphilin, respectively, stained with Coomassie blue. Lanes 4, 5, and 6, immunoblots of a duplicate of lanes 1, 2, and 3, respectively, probed with rabbit antisaxiphilin antibodies detected by peroxidase-conjugated anti-rabbit antibody and Amersham Enhanced Chemiluminescence reagents. B, Compilation of data from five SDS-Page experiments showing the relative mobility of bullfrog transferrin (■), and saxiphilin (▲) with respect to five molecular weight markers (○). Experimental details are given in Ref. (8).

The peak of  $^{55}\text{Fe}$  obtained from the DEAE column was pooled, and a sample was subjected to SDS-PAGE and stained for protein with Coomassie blue. The  $^{55}\text{Fe}$  peak contained a single major protein band (Fig. 3) that migrated with an apparent molecular weight of 78,000, consistent with the known size of serum transferrin from many vertebrates. To verify that this protein was frog serum transferrin, the iron was removed by addition of Fe chelators, 1 mM NTA and 2 mM EDTA at pH 4.2. A sample of the Fe-stripped protein was then titrated with a standard solution of 10 mM  $\text{Fe}(\text{NTA})_2$  and monitored for absorbance at 465 nm. This wavelength corresponds to a visible absorbance maximum of the  $\text{Fe}^{3+}$ -transferrin complex that gives solutions of transferrin a characteristic salmon-orange color. The results of this titration experiment, shown in Fig. 4B, indicate that  $\text{Fe}^{3+}$  binds to frog transferrin with a stoichiometry of 2  $\text{Fe}^{3+}$  ions per transferrin molecule. This Fe-binding stoichiometry is in accord with the fact that all vertebrate serum transferrins that have been isolated to date are known to contain two homologous binding sites for ferric ion. The visible absorbance spectrum was also characteristic of serum transferrin (Fig. 4A).

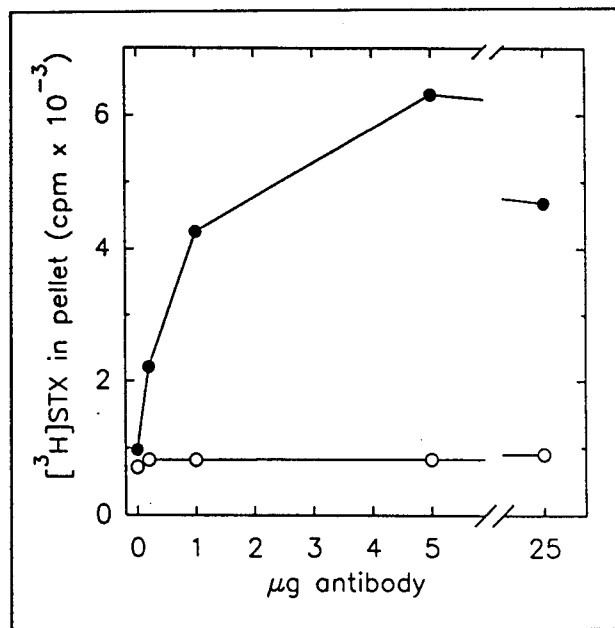




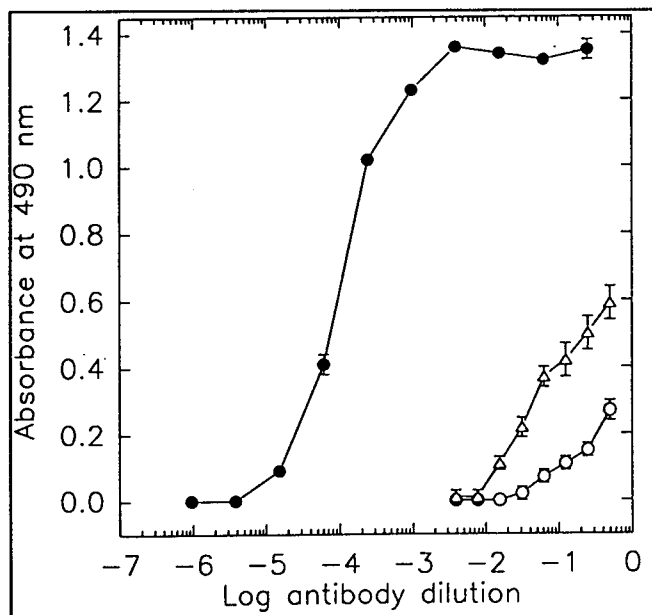
**Fig. 4.** Visible absorption spectrum and  $\text{Fe}^{3+}$  titration of bullfrog apotransferrin. A, Spectrum of 1.6 mg/ml apotransferrin before (---) and after (—) addition of  $50 \mu\text{M Fe(NTA)}_2$ . B, Absorbance change at 465 nm measured after addition of the indicated concentrations of  $\text{Fe(NTA)}_2$  to 1.6 mg/ml apotransferrin. Experimental details are given in Ref. (8).

For comparison to the isolated transferrin sample, saxiphilin was independently purified from bullfrog plasma according to the method of Li and Moczydlowski (4). When subjected to SDS-PAGE on the same gel used for analyzing transferrin, saxiphilin migrated with a distinctly larger apparent molecular weight of  $\sim 89,000$  for saxiphilin vs.  $\sim 78,000$  for transferrin (Fig. 3). To further assess the structural relationship of these two proteins, an immunochemical analysis was also performed.

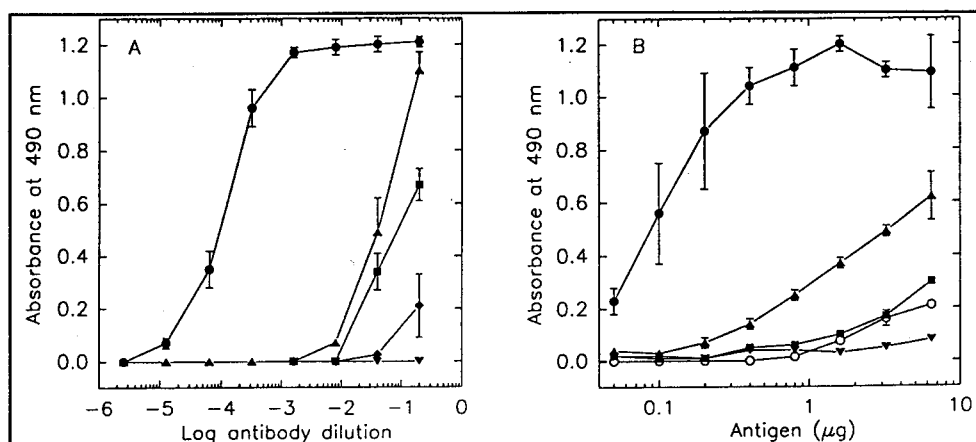
Polyclonal antibodies to native saxiphilin were raised in a rabbit by injection of bullfrog saxiphilin as an antigen. Antiserum collected from the rabbit was affinity-purified on a column of saxiphilin covalently coupled to Sepharose 4B. Antibodies prepared by this procedure were capable of immunoprecipitating  $[^3\text{H}]\text{STX}$ -binding activity from bullfrog plasma (Fig. 5), verifying their specificity for saxiphilin. These antibodies were also shown to specifically stain protein bands corresponding to saxiphilin by Western blot assay; however, there was little cross-reactivity with frog transferrin (Fig. 3A). This latter result is strong evidence that saxiphilin is a unique protein that is not directly derived from bullfrog transferrin. In a more sensitive ELISA-type assay, anti-saxiphilin antibodies exhibited about  $10^4$ -fold lower affinity for bullfrog transferrin than bullfrog saxiphilin (Fig. 6), further discriminating the two proteins. However, in the same ELISA assay, certain serum transferrins from other animal species such as cow displayed weak cross-reactivity with anti-saxiphilin antibodies (Fig. 7). Similarly, a commercial antibody against human transferrin exhibited weak cross-reactivity with bullfrog saxiphilin and several other transferrins in an analogous ELISA-assay (Fig. 8). Taken together, these results indicate that saxiphilin is not a derivative of bullfrog serum transferrin; however, weak immunochemical cross reactivity observed between saxiphilin and other species of transferrins suggests that the two proteins may share some common antigenic epitopes.



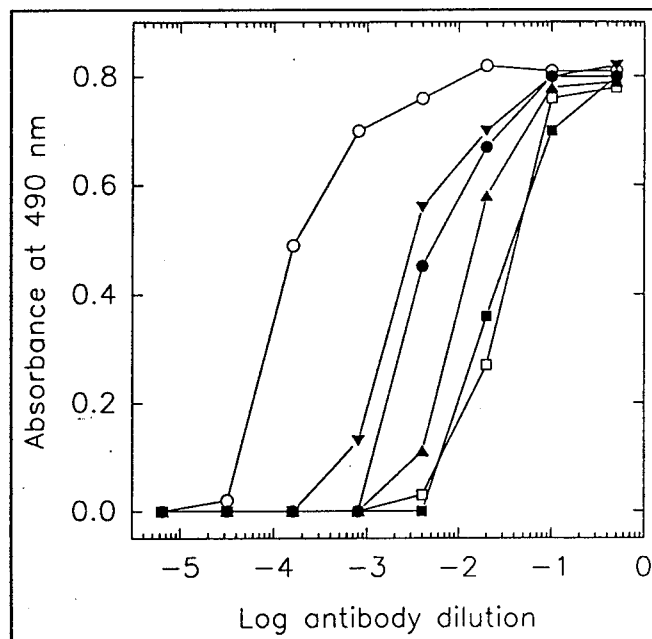
**Fig. 5.** Immunoprecipitation of [<sup>3</sup>H]STX binding activity from bullfrog plasma by antisaxiphilin antibodies. Bullfrog plasma (35 µg) was incubated with 11 nM [<sup>3</sup>H]STX in the absence (●) and presence (○) of unlabelled STX and was subjected to precipitation with increasing amounts of antisaxiphilin antibody. Experimental details are given in Ref. (8).



**Fig. 6.** Enzyme-linked immunoassay of saxiphilin and bullfrog transferrin with antisaxiphilin antibodies. Microtiter wells were coated with 250 ng of pure saxiphilin (●), bullfrog transferrin purified by chromatography on DEAE-Sephadex (Δ), or bullfrog transferrin subjected to an additional purification step of chromatography on S-Sepharose (○) and assayed using peroxidase-coupled second antibody. Experimental details are given in Ref. (8).



**Fig. 7.** Cross-reactivity of various transferrins with antisaxiphilin antibodies in an ELISA assay. (A). Microtiter wells coated with 250 ng of various antigens were incubated with increasing dilutions of antisaxiphilin antibody and assayed using peroxidase-coupled second antibody. (B). Microtiter wells coated with increasing amounts of various antigens were incubated with a 1/2000 dilution of antisaxiphilin antibody and assayed using peroxidase-coupled second antibody. (●) bullfrog saxiphilin; (▲) bovine transferrin; (■) human transferrin; (◆) horse transferrin; (▼) rabbit transferrin; (○) bullfrog transferrin after S-Sepharose chromatography. Experimental details are given in Ref. (8).



**Fig. 8.** Cross reactivity of saxiphilin and various transferrins with anti-human transferrin antibodies in an ELISA assay. Microtiter wells coated with 250 ng of various antigens were incubated with increasing dilutions of anti-human antibody and assayed using peroxidase-coupled second antibody. (○) human transferrin; (▼) rabbit transferrin; (■) chicken ovotransferrin; (▲) bovine transferrin; (■) bullfrog transferrin; (●) bullfrog saxiphilin. Experimental details are given in Ref. (8).

To examine the ligand binding activities of the two proteins, bullfrog transferrin and eight other transferrin proteins from a variety of animal species were assayed for their ability to bind [ $^3\text{H}$ ]STX. The results of these experiments (summarized in Table 1) demonstrated that binding of [ $^3\text{H}$ ]STX is a unique property of saxiphilin. Similarly, purified saxiphilin was incubated with  $^{55}\text{Fe}(\text{NTA})_2$  under conditions suitable for the binding of ferric ions to transferrin. Subsequent chromatography of this sample on a gel filtration column showed that there was no detectable association of  $^{55}\text{Fe}^{3+}$  with saxiphilin. This experiment indicates that saxiphilin does not bind iron in the manner of transferrin.

Sample	[ $^3\text{H}$ ]STX bound pmol/mg
Human apotransferrin	$0.2 \pm 0.1$
Human lactoferrin	$0.0 \pm 0.4$
Bovine apotransferrin	$-0.1 \pm 0.6$
Horse apotransferrin	$-0.1 \pm 0.3$
Rabbit apotransferrin	$0.3 \pm 0.3$
Guinea pig transferrin	$-0.1 \pm 0.3$
Mouse transferrin	$0.0 \pm 0.1$
Chicken ovotransferrin	$0.1 \pm 0.2$
Bullfrog apotransferrin (before S-Sepharose)	$12 \pm 1.1$
Bullfrog apotransferrin (after S-Sepharose)	$1.7 \pm 0.1$
Bullfrog saxiphilin	$12,000 \pm 1,200$

Table 1. Lack of [ $^3\text{H}$ ]STX binding by various transferrins. Experimental details are given in Ref. (8).

## 2. Determination of the Primary Sequence of Saxiphilin by Molecular Cloning.

Since many plasma proteins are synthesized in the liver and secreted into the plasma, we decided to use frog liver as a potential source of saxiphilin mRNA for molecular cloning. Partial amino acid sequences previously obtained by Edman degradation of tryptic fragments of saxiphilin (4) were used to design oligonucleotide primers for polymerase chain reaction (PCR) amplification. Alignment of the known partial amino acid sequences of saxiphilin with the published sequence of transferrin from the African clawed frog (*Xenopus laevis*) (11) was used in the design strategy to select appropriate sites for PCR amplification and to predict the sizes of the expected PCR products. Total RNA was isolated from bullfrog liver, and first-strand cDNA was synthesized using (dT)15 primer and murine leukemia virus reverse transcriptase. This cDNA was used as a template in a nested PCR reaction using three degenerate oligonucleotides designed to amplify a small segment of cDNA encoding for saxiphilin. This reaction yielded an ~450-bp product that was cloned into the plasmid vector pCR1000 (Invitrogen). One of the PCR clones was fully sequenced and found to contain a DNA sequence that appeared to be a promising candidate for saxiphilin sequence. This clone was then used as a hybridization probe to screen a cDNA library prepared from Poly(A)<sup>+</sup> RNA extracted from adult bullfrog liver. The cDNA library was constructed using the Lambda ZAPII cloning vector (Stratagene). Five hundred thousand recombinant phages were screened with the PCR-derived fragment. One of the positive clones isolated from the hybridization screen was found to contain a complete open reading frame of DNA encoding bullfrog saxiphilin. The DNA sequence of this clone is shown in Figure 9.



-19	Sax	MAPTFTALF	FTIISLSFAA	1	PNAKQVRWCA	a	ISDLEOKKC-	b	---NDLVGSC	b	NVPDITLVCV	a	LRSTEDCMT	a	AIKDGQADAM	*	FLDSGEVTEA	*	SKDPYNLKPI	77
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	Hstf	VAEFYGSKED	PQ-----		-----		-----		-----		-----		-----		-----		-----		-----	92
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	Sax	CPDNRTRKPLN	KYKECNLGTV	f	PAGIVVTRKI		SDKTEDINNF		LMEAQ-----		-KRO-CKLFS		SABG-KDLMF		DDSTLQALL		SSEVDAFLY-		-LGV---KLF	456
	Xltf	CPDNRTRKSIK	EYKNCNLAKV		PAHAVLTRGR		DDKSKDIIFF		LOEAQ-----		-KTQECCLFR		LPG-----MG		KGSNFGQGRS		ESYSPPIFYG		QFSVPSRLF	314
	Hstf	CLDNRTRKPEV	EYKDCHLAQV		PSHTVVARSH		GCKEDLIWEL		LNOAQEHFGK		DKSKEFQLFS		SPHG-KDLIF		KDSAHGFLKV		PPRMDAKMYL		GYE-----YV	320
	Sax	HAMKALTC--	-DAHLPSKNK	a'	-VRMCTINKL	b'	EKMKCDWDSA	b'	VSGCAIACTE	a'	ASCPKGCYVKQ	*	ILKGEADAVK	*	LEVOYMYEAL		MCGLLPAVEE		YHNKDDFGPC	552
	Xltf	QCIALKEGV	KEDDSAAQVK		-VRMCTQSKA		EKTKCDDWTT		ISGCAIEGTE		ASTAECEIVQ		ILKGDADAVT		LDGGYMYTAG		LCGLVPMVGE		YYDDDLTPC	413
	Hstf	TAIRNLREGT	CPEAPTDECK		PVKWQALSHH		ERLKCDEWSV		NSVGKIEQVS		AETTEDQIAK		INMGZADAMS		LDGGFVYIAG		KGLVFPVLAE		NYNKS--MC	418
	Sax	KTPGSPYTD	GTLRAVALVK	*	KSNKDINWNN	c'	IKGKKSCHTG	*	VGDIAGWVIP		VSLIRRONDN		SDIDSFFGES	d'	CAPGSDTKSN	e'	LCKLQICDP-	e'	KNSAANTKCS	651
	Xltf	QRSCSOAK--	GVYVAVAIVK		KG-TQVSWSN		LRGVKTCHTA		VGRTAGWNIP		VGLITSETAN		CDFASYVGES		CAPGSDVKSN		LQALGICDPE		KLSEREKKCS	510
	Hstf	ED-----TPE	AGYFAVAVVK		KSASDLTWDN		LKGKKSCHTA		VGRTAGWNIP		MGLLYNKINH		CRFDEFFSEG		CAPGSKKDSS		LCKLQMGSS-		----GLNLGE	507
	Sax	LSDKEAYYGN	OGAFRCLEVK	c'	-GDVAFVPH		VVFENTDGNK		PAVWAKNLKS	f'	EDFELLCLDG	*	SRAPVSNYKS	f'	CKLSGIPPPA	*	IVTREESISD		VVRIVANQOS	750
	Xltf	PSASEAYYGY	SGAFRCLEVK		-GOVGFARHT		TVFENTDGNK		PAGWAKDLKS		EDFELLCPDG		SRAPVTDYKR		CNLAEPVPAH		VVTLDPKREQ		VAKIVVWQOS	609
	Hstf	PNNKEGYGY	TGAFRCLEVK		-GDVAFVKGQ		TVFQNTGGKN		PDPWAKNLNE		KDYELLCLDG		TRKPVVEYAN		CHLARAPNEA		VVTRKDKEAC		VHKILRQQQH	606
	Sax	LYGRKGFE-K	DMFQLFSSNK		GNNLLFNDNT	h'	OCLITFDROP		KDIMEYFGK		PYYTIVYGAS		RSAMSSSELIS		ACTIKHC					826
	Xltf	LYGRKGFG-K	DIFQMFQSTG		GKDLLEKST		OCL--LEIPS		KTTMQEFLGD		KYHTAVTSLN		KCSTSNRASN		LPAQFHSCKM		IYMIVDCLPS	II		698
	Hstf	LFGSNVTDCS	GNFCLFERS-E		TKDLLEFRDDT		VCLAKL--HD		RNTYEKYLGE		EYKVAVGNLK		KCSTSS--LLE		ACTFRFP					679

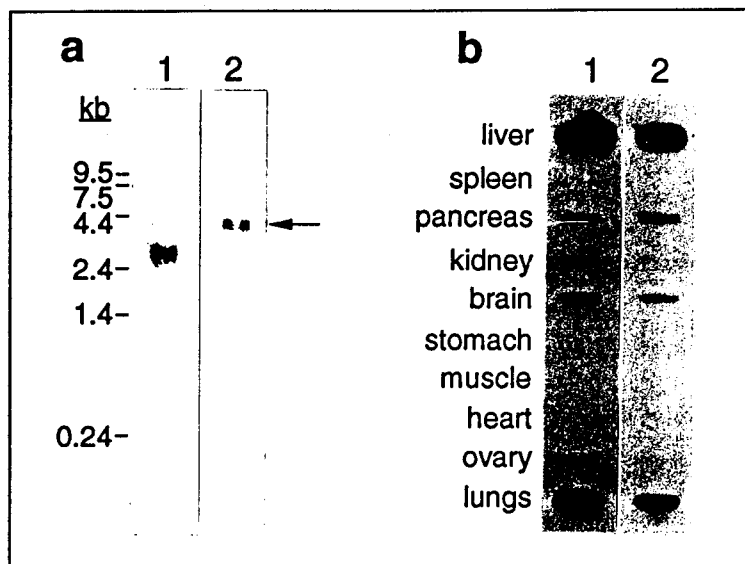
Fig. 10. Homology relationships of saxiphilin. The amino acid sequence of saxiphilin (Sax) is aligned with *X. laevis* transferrin (Xltf) and human serum transferrin (Hstf). Gaps in the alignment are shown as a hyphen (-). Residue that are identical in two of three proteins at any position are shown in boldface type. Position 1 is the N terminus and residues -19 to -1 correspond to the signal sequence. A single consensus site for N-linked glycosylation in saxiphilin is noted by an exclamation point (!). Asterisks (\*) denote the positions of 10 highly conserved residues in the two  $\text{Fe}^{3+}/\text{HCO}_3^-$  sites of transferrins. The locations of 14 probable disulfide bonds in saxiphilin were identified by homology to human lactoferrin and are labeled as *a-f* in the N-lobe and *a'-h'* in the C lobe above underlined pairs of Cys (C) residues. Experimental details are given in Ref. (9).

Figure 10 shows a protein sequence alignment of saxiphilin, serum transferrin from the *X. laevis* (11) and human serum transferrin (12) that was generated by using the PILEUP program of the Genetics Computer Group analysis package (13). Extensive sequence similarity is found throughout the whole alignment except for a 144-residue insertion that occurs after Gln-89 of saxiphilin. If this large insertion is considered as a gap, pairwise alignments between saxiphilin and the two transferrins yield values of 44% and 51% identity with human serum transferrin and *X. laevis* transferrin, respectively. Another structural feature characteristic of the transferrin family is the presence of an internal duplication (38% identity) between residues 20-487 and 488-845 of saxiphilin as detected by dot plot analysis (not shown). Similar internal duplication has been found in all previously characterized transferrins (5). This internal duplication is the basis for the 2-lobed (denoted N-lobe and C-lobe) 3-dimensional structure of transferrins as deduced for human lactoferrin (14) and rabbit serum transferrin (15) by x-ray crystallography. The transferrin protein family can also be recognized by a large number of conserved disulfide bonds. In human lactoferrin, six disulfide bonds appear at homologous positions in the N-lobe

and the C-lobe (11). In Fig. 10 these are labeled a-f and a'-f' in the putative N- and C-lobe regions of the sequence, respectively. Human lactoferrin and several other transferrins also contain four additional disulfides in the C-lobe that are not present in the N-lobe. Two of these can be identified in saxiphilin, which are labeled as g' and h' in Fig. 10. These features of the saxiphilin sequence establish its structural and evolutionary relationship to the family of transferrin proteins.

From a functional perspective, the sequence data also explain the lack of  $\text{Fe}^{3+}$  binding by saxiphilin. In most known transferrins, both the N-lobe and the C-lobe domains contain a high-affinity binding site for  $\text{Fe}^{3+}$ . X-ray crystallography and sequence analysis have previously shown that ligand residues in these two  $\text{Fe}^{3+}$ -binding sites are highly conserved (14, 15). In each lobe  $\text{Fe}^{3+}$  is coordinated by the same four residues: Asp-63 (Asp-392), Tyr-95 (Tyr 426), Tyr-188 (Tyr-517) and His-249 (His-585), with the cited sequence numbers corresponding to human serum transferrin in the N-lobe (C-lobe), respectively. Physiological binding of  $\text{Fe}^{3+}$  to these two sites in transferrin is also known to require bicarbonate anion ( $\text{HCO}_3^-$ ), which appears to bridge between  $\text{Fe}^{3+}$  and the highly conserved residue Arg-124 (Arg-456). In Fig. 10, the positions of these 10 critical residues are identified with asterisks; only one of these residues is absolutely conserved in saxiphilin at Asp-60. This lack of conservation of critical residues that form the two  $\text{Fe}^{3+}$ -binding sites in transferrin account for the fact that native saxiphilin does not appear to efficiently bind  $^{55}\text{Fe}^{3+}$  and implies that saxiphilin probably does not function in iron metabolism. The presence of the unique 144-residue insertion in saxiphilin also suggests that this domain may mediate a function that is not shared with the  $\text{Fe}^{3+}$ -binding members of the transferrin family.

To examine the expression of saxiphilin mRNA, a Northern blot of poly(A)<sup>+</sup> RNA from bullfrog liver and a slot blot analysis of total RNA extracted from various bullfrog tissues was performed. This experiment (Fig. 11) identified a ~4.4 kb RNA species in bullfrog liver that hybridized with a DNA probe specific for saxiphilin. The slot blot analysis indicated that the greatest amount of saxiphilin mRNA is present in liver, with detectable amounts also present in lung, pancreas and brain. These studies of saxiphilin RNA distribution identify the liver as an active locus of saxiphilin gene expression and synthesis. This finding is consistent with the notion that saxiphilin, like transferrin, is secreted from the liver into blood plasma where it is then available for distribution to other tissues.

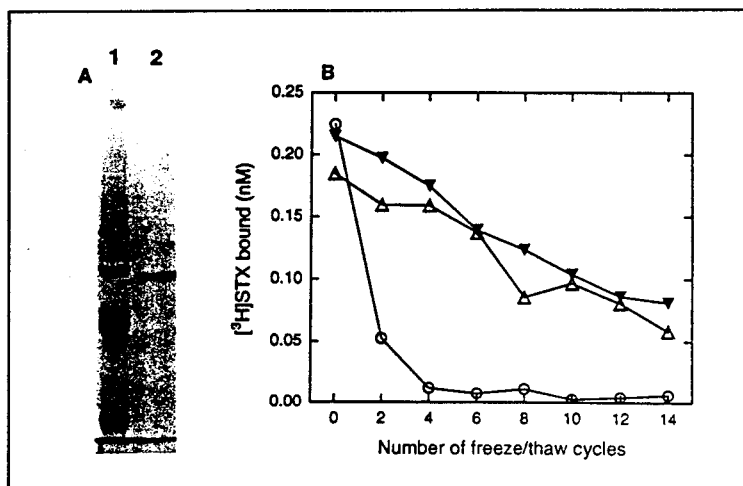


**Fig. 11.** Characterization and tissue distribution of saxiphilin mRNA. (a) Northern blot analysis of poly(A)<sup>+</sup> RNA from bullfrog liver. Five micrograms of poly(A)<sup>+</sup> RNA was fractionated on agarose, transferred to nylon, and consecutively hybridized with a 2.25 kb Sma I probe (lane 1) and a 0.43-kb insert-specific probe (lane 2). The arrow at the right points to the band identified as saxiphilin mRNA. (b) The same probes were hybridized to 5 µg of total RNA from various bullfrog tissues using a slot blot apparatus: 2.25 kb Sma I probe (lane 1); 0.43-kb insert-specific probe (lane 2). Experimental details are given in Ref. (9).

**3. Characterization of the Mechanism of Saxitoxin Binding to Purified Native Saxiphilin:** From the dual standpoint of basic and applied science, it is important to investigate the mechanism of STX binding to saxiphilin. Characterization of the kinetics of STX binding may provide insight to the function of saxiphilin in comparison to the function of transferrin. Similarly, for the application of saxiphilin in detection assays for STX and/or the development of an antidote for STX, the factors that influence toxin binding must be well understood. For this purpose, the stoichiometry, kinetics, pH-dependence, and temperature-dependence of [<sup>3</sup>H]STX binding to purified native saxiphilin have recently been characterized in detail (10). Also, the effect of various metal cations and the effect of the carboxyl group modification were also studied.

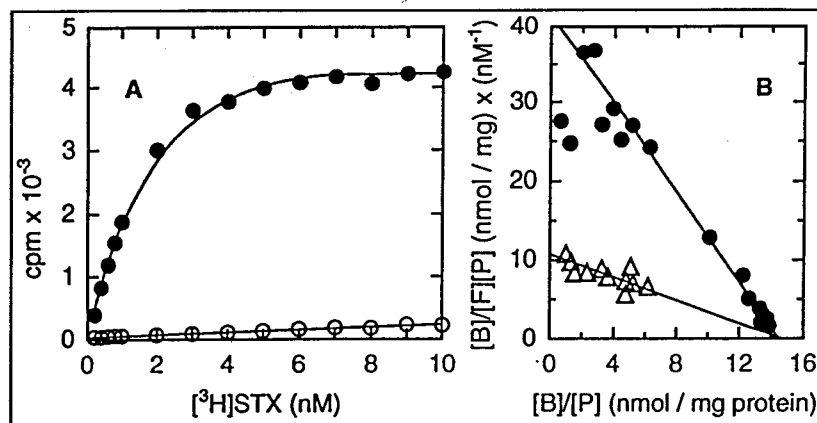
Figure 12B shows the effect of freeze-thawing on the [<sup>3</sup>H]STX binding activity of native saxiphilin. Saxiphilin is subject to denaturation by freeze-thawing as noted by complete loss of activity after four repetitive cycles of freezing in liquid N<sub>2</sub>. The addition of 20% glycerol or ethylene glycol to the freezing buffer was found to provide some protection but these cryoprotectants did not completely eliminate this form of denaturation. To minimize such loss of activity, saxiphilin was aliquoted in small volumes in buffer containing 20% glycerol and was thawed only once before use.





**Fig. 12.** Sensitivity of purified saxiphilin to freeze-thawing. (A) SDS-PAGE (7% total acrylamide) of saxiphilin used for determination of [<sup>3</sup>H]STX binding stoichiometry. Lane 1, 1.7  $\mu$ g of  $M_r$  standards (top to bottom): 116,000; 97,400; 67,000; 29,000. Lane 2, 0.8  $\mu$ g of purified saxiphilin. (B) Effect of freeze-thawing on [<sup>3</sup>H]STX binding activity of saxiphilin with no cryoprotectant (O) and partial protection by 20% glycerol (▼) or ethylene glycol (Δ). Experimental details are given in Ref. (10).

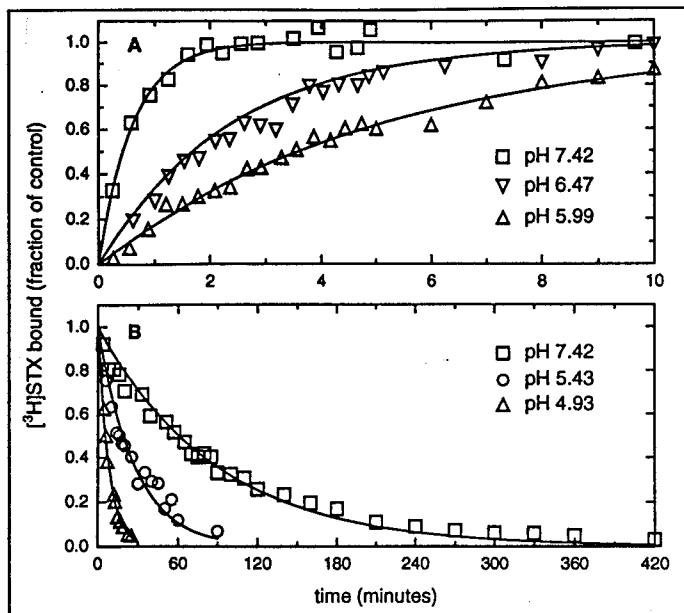
Figure 13A shows raw data from a binding titration of a pure sample of saxiphilin with increasing concentrations of [<sup>3</sup>H]STX. The low level of nonspecific binding measured in the presence of 10  $\mu$ M unlabeled STX is characteristically observed with highly purified saxiphilin. The Scatchard plot in Fig. 13B shows that [<sup>3</sup>H]STX binding to saxiphilin at pH 7.45 and 0° C is described by a homogeneous class of sites with a  $K_D$  of  $0.35 \pm 0.02$  nM and a maximal binding capacity of  $B_{\max} = 14.5 \pm 0.2$  nmol / mg protein. A similar experiment with a different preparation of saxiphilin gave values of  $K_D = 0.16 \pm 0.02$  nM and  $B_{\max} = 13.9 \pm 0.4$  nmol / mg of protein. The theoretical value of  $B_{\max}$  for a protein with a molecular mass of 90.8 kDa (as calculated from the saxiphilin primary sequence) is 11.0 nmol / mg for one STX binding site and 22.0 nmol / mg for two STX binding sites. Since the linear Scatchard plot indicates one class of sites, the data are consistent with one [<sup>3</sup>H]STX-binding site per saxiphilin molecule. This result implies that only one of the two homologous lobes of saxiphilin contains a functional STX-binding site.



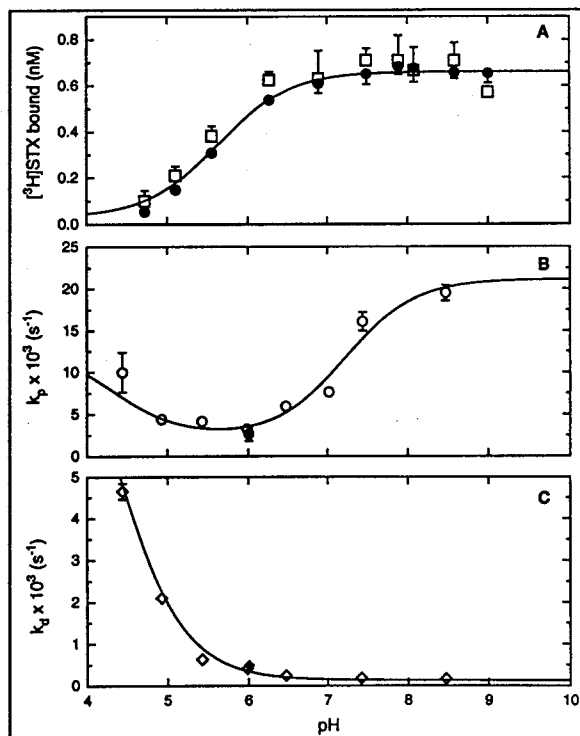
**Fig. 13.** Stoichiometry and  $K_D$  of  $[^3\text{H}]\text{STX}$  binding to pure saxiphilin. (A) Binding isotherm obtained with increasing concentrations of total  $[^3\text{H}]\text{STX}$  in the presence of 200 mM NaCl, 10 mM Mops-NaOH, pH 7.45 and 120 ng/ml saxiphilin. Samples were assayed in the absence (●) or presence of (○) 10  $\mu\text{M}$  STX to assess nonspecific binding. (B) Scatchard transformation of the data in panel A (●) is fit with a  $K_D$  of  $0.35 \pm 0.02$  nM and a  $B_{\text{max}}$  of  $14.5 \pm 0.2$  nmol/mg protein. Results are also shown for a similar experiment at pH 5.55 (Δ) and fit with a  $K_D$  of 13.7 nM and  $B_{\text{max}}$  of 14.7 nmol/mg protein. Experimental details are given in Ref. (10).

Binding of  $[^3\text{H}]\text{STX}$  to saxiphilin is inhibited by a decrease in pH. Scatchard analysis (Fig. 13B) shows that the  $K_D$  of  $[^3\text{H}]\text{STX}$  is  $13.6 \pm 3.6$  nM at pH 5.55 ( $0^\circ\text{C}$ ) which is ~40-fold lower affinity than the  $K_D$  of 0.35 nM at pH 7.45. Since the extrapolated value of  $B_{\text{max}}$  is not altered at low pH (Fig. 13B), this means that the number of available binding sites does not depend on  $[\text{H}^+]$  and that the decrease in affinity must be due to altered kinetics of  $[^3\text{H}]\text{STX}$  binding. The reduction in affinity for  $[^3\text{H}]\text{STX}$  at low pH is the combined result of a decrease of the association rate (Fig. 14A) and an increase of the dissociation rate (Fig. 14B). The time course of  $[^3\text{H}]\text{STX}$  association and dissociation followed a simple exponential function of time over a wide range of pH values, which is also consistent with the stoichiometry of one STX-binding site per saxiphilin molecule. Figure 15A is a plot of the equilibrium concentration of bound  $[^3\text{H}]\text{STX}$  as a function of pH. This experiment shows that  $[^3\text{H}]\text{STX}$  binding is essentially constant in the range of 9-7. Further acidification to pH 4 results in a progressive inhibition of binding. Fitting of the pH titration to a Hill equation gives a value of  $n = 1.0$  for the Hill coefficient and  $\text{p}K_{0.5} = 5.7$ . The Hill coefficient of 1.0 for  $\text{H}^+$  indicates that the pH-dependence of saxiphilin can be most economically explained by the participation of one ionizable group.

The data of Fig. 15A also show that the effect of pH is not due to the ionization state of the toxin. STX has two cyclized guanidinium groups as shown in the chemical structure displayed in Fig. 1. The C-2 guanidinium group of the five-membered ring of saxitoxin has a  $\text{p}K_a$  of 11.3 and the C-8 guanidinium group has a  $\text{p}K_a$  of 8.2 (16, 17). Since these guanidinium groups are completely protonated over the range of 7-4, inhibition of  $[^3\text{H}]\text{STX}$  binding by decreasing pH is most likely due to the protonation of saxiphilin. The lack of a significant decrease in the level of  $[^3\text{H}]\text{STX}$  binding in the range of pH 8-9 also implies that protonation of the C-8 guanidinium of STX is not required for high-affinity binding to saxiphilin.

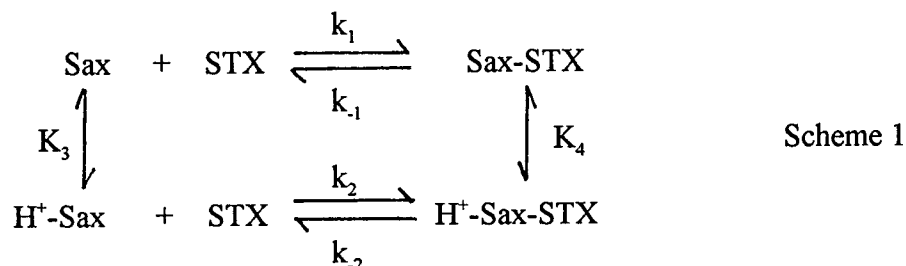


**Fig. 14.** Effect of pH on association and dissociation kinetics of  $[^3\text{H}]\text{STX}$  binding to saxiphilin. (A) Time course of  $[^3\text{H}]\text{STX}$  association at three different pH values: 5.99 ( $\Delta$ ), 6.47 ( $\nabla$ ), and 7.42 ( $\square$ ). (B) Time course of  $[^3\text{H}]\text{STX}$  dissociation was determined after equilibration for 30 minutes at the following pH: 4.93 ( $\Delta$ ), 5.43 ( $\circ$ ), and 7.42 ( $\square$ ). Experimental details are given in Ref. (10).



**Fig. 15.** Analysis of the pH dependence of  $[^3\text{H}]\text{STX}$  binding to saxiphilin (A) Equilibrium measurement of bound  $[^3\text{H}]\text{STX}$  after incubation of 5 nM  $[^3\text{H}]\text{STX}$  at pH 9 to 4 in the presence of 200 mM NaCl ( $\bullet$ ). Results for a similar experiment at higher ionic strength (600 mM NaCl) are also shown ( $\square$ ). (B) Apparent pseudo-first order rate constant for association of  $[^3\text{H}]\text{STX}$  as a function of pH. (C) Apparent rate constant for dissociation of  $[^3\text{H}]\text{STX}$  as a function of pH. Experimental details are given in Ref. (10).

To investigate the detailed kinetic mechanism underlying the pH-dependence of [ $^3\text{H}$ ]STX binding, the association and dissociation rate constants for this process were measured at various pH values from 9 to 4.7. The results in Fig. 15B show that the pseudofirst order association rate constant for [ $^3\text{H}$ ]STX follows a biphasic dependence on pH with an apparent minimum near pH 5.5. The result in Fig. 15C shows that the dissociation rate constant increases in a monotonic fashion with decreasing pH. Analysis of these results revealed that the pH dependence of [ $^3\text{H}$ ]STX binding to saxiphilin can be quantitatively described by the following model where Sax refers to the unprotonated form of saxiphilin and  $\text{H}^+$ -Sax refers to the protonated form:



In the above scheme,  $k_1$  and  $k_2$  are the bimolecular association rate constants for STX binding to the unprotonated and protonated forms of saxiphilin, respectively. The dissociation constants,  $k_{-1}$  and  $k_{-2}$  are the respective rate constants for STX dissociation.  $K_3$  and  $K_4$  are the equilibrium dissociation constants for protonation of the STX-free and STX-bound forms of saxiphilin, respectively. To determine whether Scheme 1 is sufficient to describe the pH-dependence of [ $^3\text{H}$ ]STX binding, the data in Figs. 15A, B and C were simultaneously fit to three equations derived from Scheme 1 using five independent kinetic parameters:  $k_1$ ,  $k_{-1}$ ,  $k_2$ ,  $k_{-2}$  and  $K_4$ . The results of the fitting procedure are shown as solid lines in Fig. 15 with the best-fit parameters listed in Table 2. The agreement between the data and the model shows that Scheme 1 provides a good description of the pH-dependence of STX binding. In terms of a molecular interpretation, Scheme 1 implies that the pH-dependence of STX binding is mediated by a single titratable residue of saxiphilin. The unprotonated form of saxiphilin binds STX with high affinity ( $K_D = K_1 = 0.12$  nM). Protonation of the STX-free form of saxiphilin with a  $\text{pK}_a$  of  $\text{pK}_3 = 7.2$  results in an  $\text{H}^+$ -form of the protein that has lower affinity for STX ( $K_2 = 103$  nM). Shifting the equilibrium to the species,  $\text{H}^+$ -Sax, thus results in a lower apparent association rate. Similarly, protonation of the STX-bound form of saxiphilin with a  $\text{pK}_a$  of  $\text{pK}_4 = 4.3$  also destabilizes STX binding and results in an  $\text{H}^+$ -saxiphilin-STX ternary complex that has a faster dissociation rate of STX, giving rise to pH-dependent dissociation of [ $^3\text{H}$ ]STX.

parameter	value	units
$k_{-1}$	$1.3 \pm 1.0 \times 10^{-4}$	$\text{s}^{-1}$
$k_1$	$1.1 \pm 0.1 \times 10^6$	$\text{s}^{-1} \text{M}^{-1}$
$k_{-2}$	$1.1 \pm 0.9 \times 10^{-2}$	$\text{s}^{-1}$
$k_2$	$1.1 \pm 0.3 \times 10^5$	$\text{s}^{-1} \text{M}^{-1}$
$K_1$	$1.2 \times 10^{-10}$	M
$K_2$	$1.0 \times 10^{-7}$	M
$K_3$	$6.0 \times 10^{-8}$	M
$K_4$	$5.1 \pm 0.6 \times 10^{-5}$	M

**Table 2.** Binding parameters for a 4-state model of pH-dependent binding of STX to saxiphilin. Details of the analysis are given in Ref. (10).

The temperature-dependence of [ $^3\text{H}$ ]STX binding was investigated by measuring the dissociation rate constant,  $k_d$ , and the equilibrium  $K_D$  at  $5^\circ$  intervals over the temperature range of  $0^\circ\text{C}$  to  $30^\circ\text{C}$  at pH 7.4. The apparent bimolecular association rate constant at these temperatures was obtained from the relationship,  $k_a = k_d/K_D$ . The results are presented as a combined van't Hoff plot ( $\ln K_D$  vs.  $T^{-1}$ ) and Arrhenius plots ( $\ln k$  vs.  $T^{-1}$ ) in Fig. 16. These plots are well-described by linear functions in the temperature range of  $0^\circ$ - $25^\circ\text{C}$ . At  $30^\circ\text{C}$  the measured  $K_D$  deviated from linear behavior suggesting either a temperature-dependent transition or possibly thermal inactivation. Of the three parameters,  $k_d$  exhibited the highest temperature dependence with a 4.3-fold increase from  $0^\circ$  to  $10^\circ\text{C}$ . For this same temperature increase,  $k_a$  and  $K_D$  increased by 1.7-fold and 2.2-fold, respectively. The solid line through the  $K_D$  values in Fig. 15 is a fit to the equation,  $\ln K_D = \Delta H^\circ/RT - \Delta S^\circ/R$ , using  $\Delta H^\circ = -8.3 \pm 2.0\text{ kcal mol}^{-1}$  and  $\Delta S^\circ = 13.8 \pm 7.2\text{ cal mol}^{-1}\text{ K}^{-1}$ . The solid lines through the  $k_d$  and  $k_a$  values are fit to the equation,  $\ln k = \ln A - E_a/RT$  using  $E_a = 22.5 \pm 1.0\text{ kcal mol}^{-1}$  for  $k_d$  and  $11.1 \pm 2.1\text{ kcal mol}^{-1}$  for  $k_a$ . At  $0^\circ\text{C}$ ,  $T\Delta S^\circ$  is equal to  $3.8\text{ kcal mol}^{-1}$ , which indicates that the enthalpic term predominates over the entropic term in the free energy change of binding ( $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$ ).

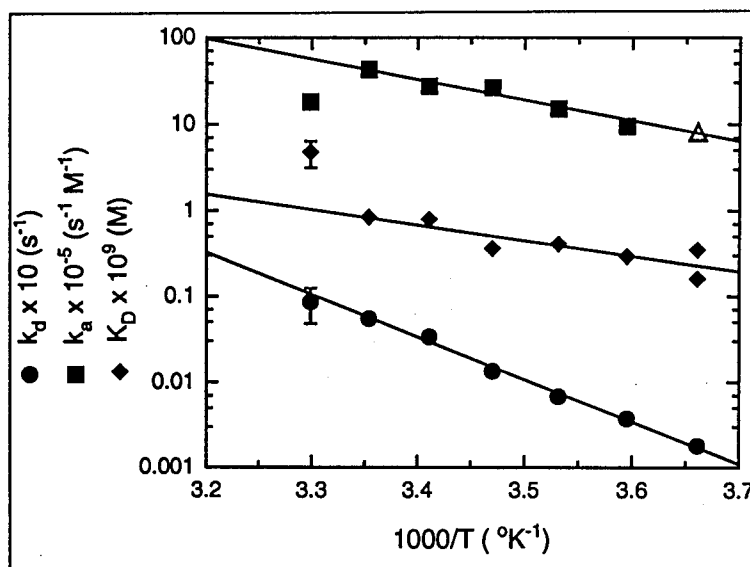


Fig. 16. Arrhenius and van't Hoff plots of [ $^3\text{H}$ ]STX binding to saxiphilin at pH 7.4.  $K_D$  values ( $\blacklozenge$ ) were determined from Scatchard analyses performed at  $5^\circ\text{C}$  intervals between  $0^\circ$  and  $30^\circ\text{C}$ .  $k_d$  ( $\bullet$ ) values were similarly determined from the dissociation time course at various temperatures.  $k_a$  values ( $\blacksquare$ ) were calculated from the measured  $k_d$  and  $K_D$  values according to  $k_a = k_d/K_D$ . Experimental details are given in Ref. (10).

By all indications, binding of [ $^3\text{H}$ ]STX to saxiphilin is a chemically specific interaction. The only class of organic molecules found to competitively inhibit this binding reaction are STX and naturally occurring or synthetic derivatives of STX that differ by small chemical substituents (3). In the course of the present experiments, we found that a variety of other molecules that structurally resemble STX, such as adenosine, 8-aminoguanine, 8-aminoguanosine, creatinine, folic acid, uric acid, and xanthine, do not affect [ $^3\text{H}$ ]STX binding to saxiphilin when tested at

concentrations in the range of 1-10 mM. Such observations emphasize that the structural requirements for ligand binding to saxiphilin are stringent.

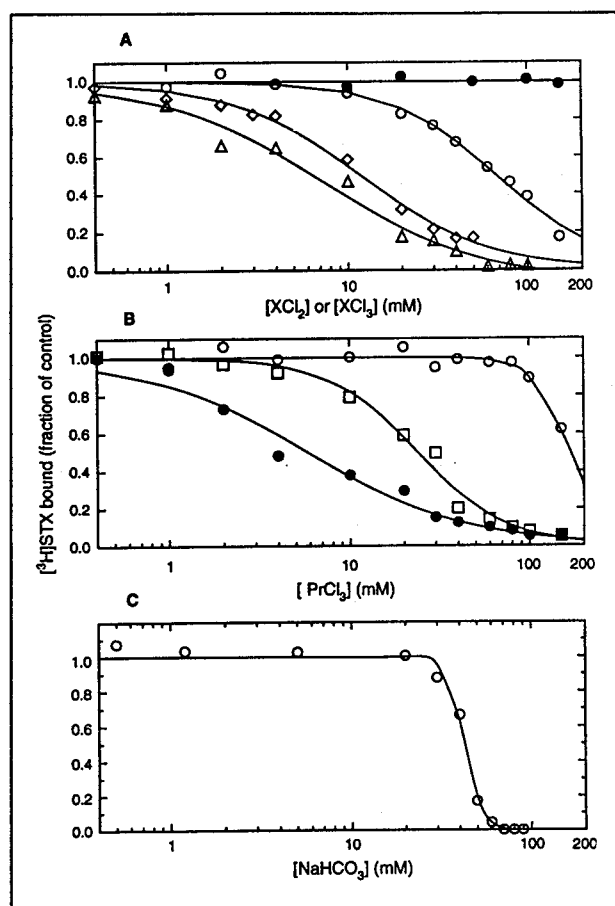
Since saxiphilin is homologous to transferrin, an important question is whether it can bind metal ions. Thus far, attempts to directly demonstrate transferrin-like binding of  $^{55}\text{Fe}^{3+}$  have been unsuccessful (8). To further examine this question, various divalent metal and lanthanide cations were tested for their ability to inhibit binding of  $[^3\text{H}]\text{STX}$  both in the presence and absence of  $\text{NaHCO}_3$ . As summarized in Figs. 17 and 18, a variety of such metal ions display inhibition in the millimolar range. For example, Figure 17A shows that  $\text{Zn}^{2+}$  inhibits  $[^3\text{H}]\text{STX}$  binding with a  $K_{0.5}$  of 67 mM. Such inhibition is not merely an effect of ionic strength since  $\text{MgCl}_2$  tested up to 200 mM (Fig. 17A) and  $\text{NaCl}$  tested up to 1 M (not shown) has no effect on the control level of  $[^3\text{H}]\text{STX}$  binding. Furthermore, the data of Figs. 17 and 18 show that certain lanthanide cations such as  $\text{Eu}^{3+}$ ,  $\text{Tb}^{3+}$ , and  $\text{Nd}^{3+}$  are more effective inhibitors of  $[^3\text{H}]\text{STX}$  binding than the tested transition metals ( $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ ) with a  $K_{0.5}$  of  $\sim 6$  mM. This inhibition is not strictly dependent on added  $\text{HCO}_3^-$  as noted by the similar  $K_{0.5}$  values measured with or without 100 mM  $\text{NaHCO}_3$  (Fig. 18). However, several of the lanthanides such as  $\text{Tb}^{3+}$ ,  $\text{Pr}^{3+}$  and  $\text{La}^{3+}$  do show a 2- to 10-fold enhancement of affinity in the presence of  $\text{HCO}_3^-$ . Some of the metal ions exhibited Hill coefficients ( $n$ ) greater than 1.0 in displacement titrations, suggesting the participation of more than one ion (e.g.,  $n = 1.5$  for  $\text{ZnCl}_2$ , Fig. 17A). However, it is possible that such behavior may also be related to slow equilibration as described below for  $\text{Pr}^{3+}$ .

In the case of  $\text{Pr}^{3+}$ , an unusual phenomenon was observed. If saxiphilin is incubated for 1 hr with  $[^3\text{H}]\text{STX}$  and increasing concentrations of  $\text{Pr}^{3+}$ , practically no binding inhibition is observed with up to 100 mM  $\text{PrCl}_3$  in the absence of added  $\text{HCO}_3^-$  (Fig. 17B). However, effective displacement by  $\text{Pr}^{3+}$  ( $K_{0.5} = 6$  mM) is observed in the presence of 100 mM  $\text{HCO}_3^-$  (Fig. 17B). When assayed after equilibration for 1 hr,  $\text{NaHCO}_3$  promotes the inhibition of  $[^3\text{H}]\text{STX}$  binding in the presence of 100 mM  $\text{Pr}^{3+}$  in a concentration-dependent fashion (Fig. 17C). The unusually steep nature of the  $\text{HCO}_3^-$  titration curve (Fig. 17C) suggested that the effect of  $\text{HCO}_3^-$  might reflect a synergistic effect on the rate at which  $\text{Pr}^{3+}$  is able to displace  $[^3\text{H}]\text{STX}$  rather than highly cooperative binding of  $\text{HCO}_3^-$ . Indeed, if  $\text{Pr}^{3+}$  is incubated for 6 hr with saxiphilin and  $[^3\text{H}]\text{STX}$  in the absence of  $\text{HCO}_3^-$ , the titration curve for  $\text{Pr}^{3+}$  approaches that observed for a 1 hr incubation with 100 mM  $\text{HCO}_3^-$  (Fig. 17B). These results indicate that equilibration of  $\text{Pr}^{3+}$  with saxiphilin is slow in the absence of  $\text{HCO}_3^-$  and that  $\text{HCO}_3^-$  acts synergistically to enhance the rate of equilibration of the metal ion.

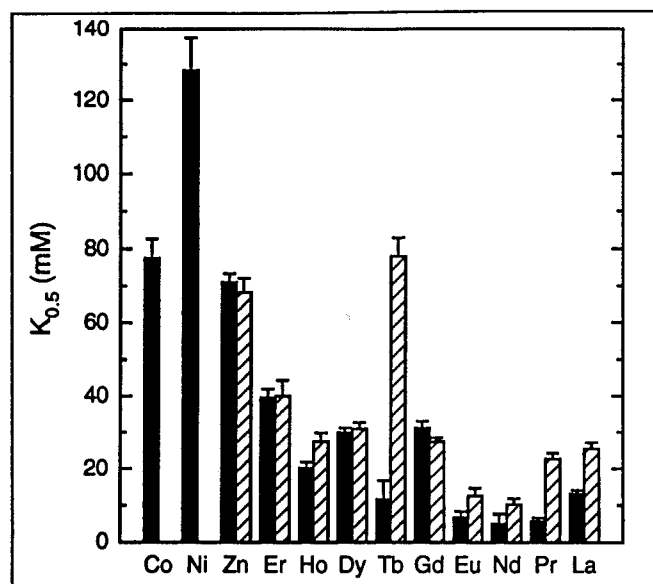
In the presence of 10 mM  $\text{PrCl}_3$  and 100 mM  $\text{NaHCO}_3$ , Scatchard analysis revealed a low affinity  $K_D$  of 4.6 nM for  $[^3\text{H}]\text{STX}$  binding without a change in  $B_{\text{max}}$ , indicative of a competitive interaction between the toxin and the lanthanide cation (data not shown). To further examine the mechanism of inhibition of  $[^3\text{H}]\text{STX}$  binding by lanthanide cations, the effect of 10 mM  $\text{Pr}^{3+}$  plus 100 mM  $\text{NaHCO}_3$  on the time course of  $[^3\text{H}]\text{STX}$  association and dissociation was studied. The results of Fig. 19 show that the reduced affinity for  $[^3\text{H}]\text{STX}$  in the presence of  $\text{Pr}^{3+}/\text{HCO}_3^-$  is due to a large decrease in the toxin association rate with virtually no effect on the toxin dissociation rate. Thus, inhibition of  $[^3\text{H}]\text{STX}$  binding by lanthanides is very different from that of  $\text{H}^+$ , which greatly accelerates the dissociation rate as well as slowing the association of  $[^3\text{H}]\text{STX}$ . The results of Fig. 19 are consistent with a model in which a low-affinity metal cation-binding site is

formed by amino acid residues that directly participate in STX binding. If binding of a lanthanide ion and STX are mutually exclusive,  $\text{Pr}^{3+}$  only would affect the association rate of  $[^3\text{H}]\text{STX}$  and not the toxin dissociation rate, since the two ligands would never be simultaneously bound.

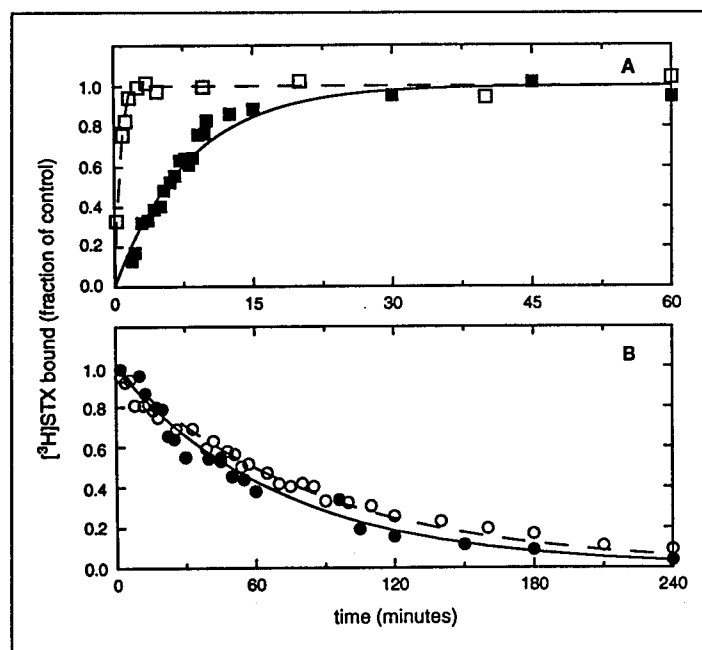
Trimethylloxonium (TMO) is a protein modification reagent that methylates carboxyl groups of aspartate and glutamate residues in a rather specific manner (18, 19). As shown in Fig. 20, treatment of saxiphilin for 10 min with increasing concentrations of TMO resulted in the complete inhibition of  $[^3\text{H}]\text{STX}$  binding. However, when the pre-bound complex of saxiphilin and  $[^3\text{H}]\text{STX}$  was treated with TMO in the same fashion, virtually complete protection was observed (Fig. 20). These results suggest that carboxyl groups of saxiphilin are involved in STX binding. It is likely that the observed inhibition of  $[^3\text{H}]\text{STX}$  binding by TMO results from the methylation of aspartate and/or glutamate residues of saxiphilin that participate in hydrogen bonds with the bound toxin molecule.



**Fig. 17.** Inhibition of  $[^3\text{H}]\text{STX}$  binding to saxiphilin by various divalent and trivalent cations. (A) Effect of ( $\Delta$ )  $\text{EuCl}_3$ , ( $\diamond$ )  $\text{TbCl}_3$ , (O)  $\text{ZnCl}_2$ , and ( $\bullet$ )  $\text{MgCl}_2$  on equilibrium binding of  $[^3\text{H}]\text{STX}$ . Data are normalized to the control value in the absence of the metal ions. (B) Effect of  $\text{PrCl}_3$  measured after a 1 h incubation (O), effect of  $\text{PrCl}_3$  measured after a 6 h incubation ( $\square$ ), and effect of  $\text{PrCl}_3$  plus 100 mM  $\text{NaHCO}_3$ . (C) Titration of  $\text{NaHCO}_3$  in the presence of 100 mM  $\text{PrCl}_3$ , assayed after 1 h incubation. Experimental details are given in Ref. (10).



**Fig. 18.** The concentration ( $K_{0.5}$ ) of various divalent metal and lanthanide cations that produced 50% inhibition of  $[^3\text{H}]\text{STX}$  binding as determined in Figure 17. Solid bars correspond to results obtained in the presence of  $100 \text{ mM NaHCO}_3$ , and cross-hatched bars were obtained in the absence of  $\text{NaHCO}_3$ . Experimental details are given in Ref. (10).



**Fig. 19.** Effect of  $\text{PrCl}_3$  plus  $\text{NaHCO}_3$  on the kinetics of  $[^3\text{H}]\text{STX}$  binding to saxiphilin. The association time course (A) and the dissociation time course (B) of  $[^3\text{H}]\text{STX}$  binding was determined in the absence (open symbols) or presence (filled symbols) of  $10 \text{ mM PrCl}_3$  plus  $100 \text{ mM NaHCO}_3$ . Experimental details are given in Ref. (10).



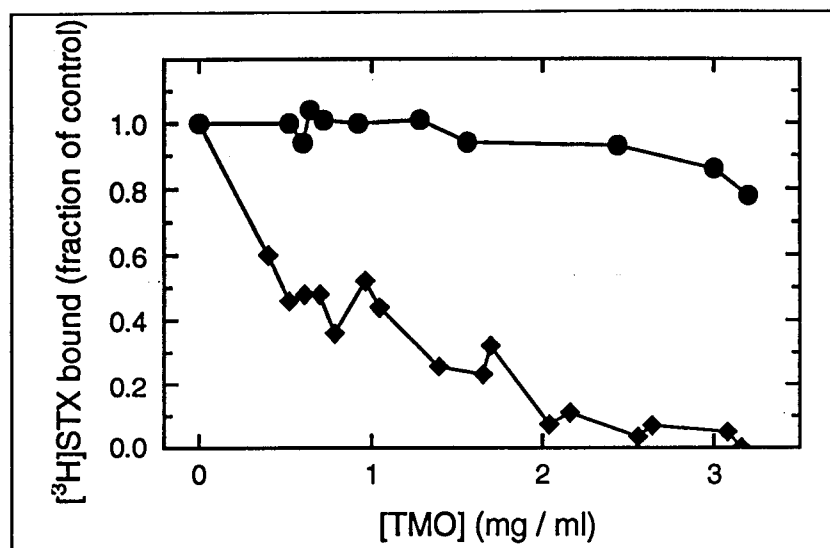
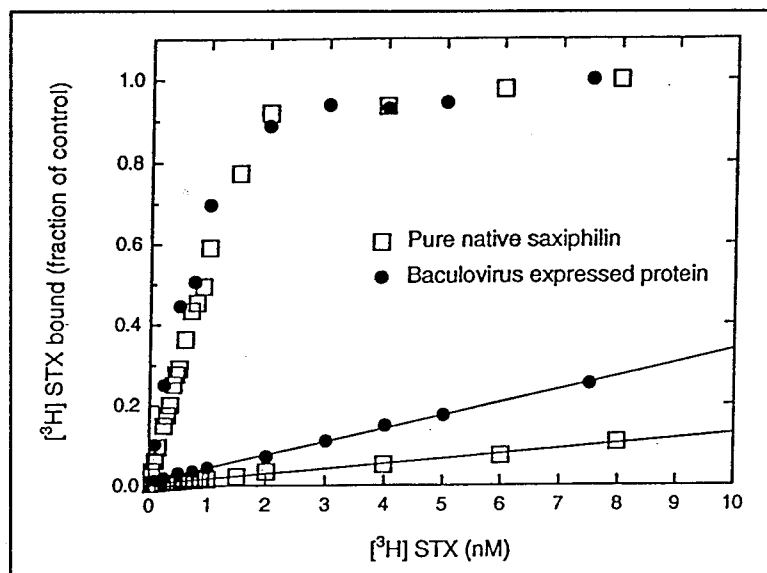


Fig 20. Effect of chemical modification by TMO on  $[^3\text{H}]\text{STX}$  binding to saxiphilin. Saxiphilin was pre-exposed to the indicated concentrations of TMO for 10 min in the absence (●) or presence (◆) of 19.2 nM  $[^3\text{H}]\text{STX}$  before assay of  $[^3\text{H}]\text{STX}$  binding. Data are normalized to the value in the absence of TMO. Experimental details are given in Ref. (10).

**4. Functional Expression of Recombinant Saxiphilin and a Bioengineered Fragment of Saxiphilin.** We first attempted to express saxiphilin in *E. coli* using the pMAL expression vector from New England Biolabs. With this system, foreign proteins are expressed as a fusion protein of the bacterial maltose-binding protein. Although we did obtain antibody-reactive products, the resulting fusion protein exhibited a lower molecular mass than expected, indicating that it was extensively degraded. Also, there was no suggestion of functional activity as detected by  $[^3\text{H}]\text{STX}$ -binding assay. These results suggested that *E. coli* is not an appropriate organism for expression of saxiphilin. The exact reasons for this are unknown, but it may be due to differences in the mechanisms of protein folding and secretion in bacteria versus eucaryotic cells.

We next decided to attempt to express saxiphilin in cultured insect cells (Sf9 cell line) using the baculovirus-mediated expression system. The cloned saxiphilin gene was inserted into the pBlueBac III transfer vector obtained from Invitrogen. This vector was used to co-transfect insect Sf9 cells together with wild-type baculovirus in order to obtain a recombinant virus containing the saxiphilin gene linked to the polyhedrin promoter. This experiment was successful as indicated by the appearance of  $[^3\text{H}]\text{STX}$ -binding activity in the culture supernatant of the co-transfected cells. We have since isolated viral clones that are capable of directing the expression of saxiphilin, which is secreted into the medium by infected insect cells. Figure 21 shows a comparison of  $[^3\text{H}]\text{STX}$  binding to recombinant saxiphilin and native saxiphilin. The results of such binding studies indicate that the functional properties of recombinant saxiphilin produced in this manner are virtually identical to those of native saxiphilin. The molecular mass of the recombinant protein (~91 kDa) is also virtually the same as the native protein as detected by Western blot analysis (data not shown). We are now in the process of developing methods for the large scale expression and purification of recombinant saxiphilin. At present, the potential yield of saxiphilin is about 2 mg of protein per liter of culture supernatant. Since *in vivo* studies

on the protective effects of saxiphilin will potentially require hundreds of milligrams of protein, we are currently examining strategies to make the production of saxiphilin more efficient and less expensive.



**Fig. 21.** Titration of [<sup>3</sup>H]STX binding to native saxiphilin (□) and recombinant saxiphilin (●) expressed in cultured insect Sf9 cells. The data points connected with solid lines correspond to duplicate assays in the presence of 10 μM STX to assess non-specific binding.

Recently, we achieved a breakthrough in localization of the STX binding site. Based on comparison of sequence differences between saxiphilin and transferrins, we postulated that the STX binding site is located in the C-lobe domain of the protein. To test this idea, we engineered a baculovirus expression vector which codes for the complete C-lobe of saxiphilin with the N-lobe domain deleted (residues 2-465). Subsequent infection of insect cells with this vector resulted in the secretion of [<sup>3</sup>H]STX-binding activity into the medium confirming that the STX binding site is indeed located in the C-lobe. Studies currently in progress show that the C-lobe protein binds STX nearly as well as the native protein. The measured  $K_D$  for [<sup>3</sup>H]STX binding to the C-lobe is ~0.8 nM as compared to a  $K_D$  of ~0.2 nM for the whole protein. This finding means that the size of the STX-binding protein has been reduced from 91 kDa of native saxiphilin to 40 kDa for the C-lobe protein. This truncated C-lobe protein may also be useful for applications aimed at detecting and counteracting the toxic effects of STX. We are currently in the process of preparing these results for publication.

**5. Phylogenetic Distribution of Saxiphilin-like Activity and the Comparative Pharmacology of Saxiphilin from Other Animal Species (Preliminary Results):** As an approach toward uncovering the biological function of saxiphilin, we have recently carried out an extensive survey of the distribution of saxiphilin-like activity in various classes of animals. The rationale for this study is that knowledge of the distribution of saxiphilin within the global biological kingdom may reveal correlations that lead to testable hypotheses regarding function. The comparative biochemistry of saxiphilin from animals other than frogs may also identify

saxiphilin variants with more desirable properties as a detection agent or antidote. Most of the data generated from this work are still being analyzed and are not yet ready for publication. However, our basic finding is that saxiphilin-like activity (defined as soluble, high-affinity [ $^3\text{H}$ ]STX-binding activity) is present in a wide variety of fish, amphibians and reptiles of diverse habitat and geographical distribution. This indicates that the saxiphilin gene is widely distributed in the evolutionary tree. However, we have not yet detected saxiphilin-like activity in birds, mammals or marine fish. Also, we have not detected saxiphilin-like activity in invertebrate species such as shellfish that are known to be exposed to STX. Further work is needed to determine whether the saxiphilin gene is absent in animals that lack soluble STX-binding activity or whether its expression is regulated. Preliminary results also reveal that the pharmacology of binding of various STX derivatives can vary substantially among different animals. For example, saxiphilin from *Rana* frogs appears to be unusual in that it binds neosaxitoxin with 500-fold lower affinity than STX. Saxiphilin activity from many other species such as garter snakes exhibits nearly the same high affinity for neosaxitoxin and STX. We have also found that certain species of saxiphilins have a much slower dissociation rate for [ $^3\text{H}$ ]STX than frog saxiphilin. These latter properties may provide advantages in certain applications of saxiphilin as a detection reagent for STX.

**CONCLUSIONS:** The work carried out thus far under this project has produced a number of original findings and accomplishments as listed below.

1. Saxiphilin is not a derivative of transferrin but is a unique member of the transferrin superfamily of proteins. It does not appear to function in iron transport or metabolism as judged by substitution of nearly all of the conserved residues of transferrin that coordinate directly with  $\text{Fe}^{3+}$  and a lack of detectable  $^{55}\text{Fe}^{3+}$  binding.
2. The complete primary amino acid sequence of bullfrog saxiphilin has been obtained by molecular cloning. This sequence indicates that saxiphilin is secreted from frog liver as an 826-residue polypeptide after cleavage of a 19-residue signal sequence. Sequence alignment with transferrins whose structures have been solved by x-ray diffraction can be used to infer that saxiphilin contains at least 14 disulfide bonds.
3. Binding titration of pure native saxiphilin with [ $^3\text{H}$ ]STX shows that there is one high affinity STX-binding site per molecule with a  $K_D$  of  $\sim 0.2$  nM at  $0^\circ\text{C}$  and pH. 7.4.
4. Binding of STX to saxiphilin is inhibited by decreasing pH with half-maximal inhibition occurring at pH 5.7. The inhibition of STX binding by  $\text{H}^+$  is the combined result of a slower association rate and a faster dissociation rate. The pH-dependent kinetics of STX binding can be explained by an allosteric model in which protonation of a single titratable residue results in a low affinity conformation of saxiphilin with respect to STX.
5. The temperature dependence and thermodynamics of [ $^3\text{H}$ ]STX binding to saxiphilin have been characterized. Since the equilibrium  $K_D$  for [ $^3\text{H}$ ]STX is in the nanomolar range at  $37^\circ$

C, the protein may potentially be useful for small mammal *in vivo* studies aimed at exploring its effectiveness in protecting against the toxic effects of STX.

6. Studies using trivalent lanthanide cations as probes of the STX binding site and chemical modification experiments with trimethyloxonium (TMO) suggest that the STX-binding site of saxiphilin contains one or more carboxylate residues. This information together with localization of the STX-binding activity to the C-lobe domain may be useful in the identification of specific residues that form the toxin binding site.

7. The cloned gene for saxiphilin has been inserted into a baculovirus expression vector that directs the synthesis of functionally active protein in cultured insect cells. A similar baculovirus engineered to express a 40-kDa fragment of saxiphilin corresponding to the C-lobe domain also yielded [<sup>3</sup>H]STX-binding activity, showing that the STX-binding site is located in this portion of the protein molecule. These results are an important first step toward the development of high-level expression of recombinant saxiphilin.

8. Saxiphilin-like activity has been detected in a number of freshwater fish, amphibians and reptiles, indicating that the saxiphilin gene is widely distributed throughout the vertebrate evolutionary tree.

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